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SUBMARINE ATMOSPHERES

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SUMMARY

Nuclear submariners live and work in an atmosphere composed of approximately 80% naturally occurring nitrogen, 19% oxygen (manufactured aboard ship), and a complex mixture of inorganic and organic contaminants. The concentrations of contaminants exist as a balance between the rates of production from human and operational activities and the rate of removal by engineering systems. The biological effects of inorganic gases, particularly carbon dioxide, have been extensively studied. Investigators are now attempting to define the composition and concentration of volatile organic compounds that accumulate during 90-day submergences. Medical studies have not conclusively shown that crewmembers incur adverse health effects from continuous exposures to the sealed atmospheres of nuclear submarines.

HISTORICAL REVIEW

Vast improvements in propulsion plants and air purification systems have effectively lowered the risk of acute intoxication from exposure to submarine atmospheres. World War I crews risked acute poisoning by exposure to the fumes of gasoline engines and batteries in submarines that were not equipped with air reclamation systems. The submergence time of these ships was limited by the environmental effects of human metabolism, which raised the percentage of atmospheric carbon dioxide (CO_2) and lowered the percentage of atmospheric oxygen (O_2) in approximately a 1:1 ratio. Crewmembers experienced symptoms of breathlessness after 8 to 17 h of respiration changed their unprocessed and stagnant atmosphere to 15% O_2 and 5% CO_2. Odors were particularly annoying due to the inadequate facilities for personal hygiene and the lack of air purification devices [1,2].

During the South Pacific operations of World War II crewmembers were menaced with labored breathing, headaches, dehydration, and syncope during submergences in diesel-electric submarines that extended beyond the daytime period. The standard procedure for revitalizing the atmosphere was to ventilate the ship with fresh air at nighttime in order to sustain the metabolic requirements for oxygen during daytime submergences. The submarines were not equipped with air conditioning systems for dissipating excess heat into the tropical oceans, and lithium hydroxide (LiOH) was only used to scrub excess CO_2 from the atmosphere in emergency situations. Oxygen deficiency was detected by the inability to light cigarettes rather than by routine monitoring of the ship's atmosphere. Each submarine carried a limited number of chlorate candles to provide the crew an

emergency source of O_2. Charcoal filters helped improve the atmosphere by partially removing annoying odors, but the greatest improvement in habitability occurred toward the end of the War when advanced systems were installed to recirculate the air through air conditioning plants [3-5].

As a prelude to the deployment of nuclear submarines, it was necessary for physiologists to define the concentrations of CO_2 for safe habitation. The use of LiOH to remove CO_2 was deemed impractical due to the excessive quantity of chemicals needed for long patrols. Therefore, new machinery was designed to absorb CO_2 with recycled monoethanolamine [2,6,7].

The first nuclear submarine, the USS Nautilus, set a submergence record of 11 days. It was during this habitability cruise in 1956 that chemists discovered a variety of different hydrocarbons in high atmospheric concentrations. (This proved to be of more than academic interest as there were instances in later submarine patrols when the irritating effects of atmopsheric contaminants interfered with crew performance.) The progressive accumulation of pollutant gases during multiday submergences established the need to minimize the rate of release of chemical vapors into the atmosphere while at the same time maintaining an acceptable rate of removal. The Navy published a restricted items list for the exclusion or limitation of organic solvents and toxic chemicals from submarines. Hopcalite® was placed in catalytic burners to accelerate the thermal conversion of carbon monoxide, hydrogen, and hydrocarbon molecules to CO_2 and water [2,8]. The length of submergences was now limited by the shipboard supply of O_2 for metabolic consumption. The USS Seawolf used auxiliary stores of O_2 to sustain its crew during a 60-day submergence. Later classes of nuclear submarines were fitted with generators that manufactured O_2 by the electrolysis of water [2]. The nuclear submarines also were equipped with gas analyzers to monitor the composition of the atmosphere on a continual basis.

Today's submarine atmospheres are still contaminated with trace concentrations of many organic compounds. This is illustrated in Figure 1, which shows the ion chromatograms of equal-volume air samples taken from a submarine's atmopshere (upper panel) and a clean office (lower panel). The submarine air contained more compounds at higher concentrations than did office air. These compounds were volatile organic compounds (VOCs) that consisted of long chain aliphatic hydrocarbons, aromatic compounds, and halocarbons. The list of organic pollutants currently identified aboard nuclear submarines is too long for this discussion; however, reviews by Carhart and Johnson [6] and the National Academy of Sciences [9,10] provide interesting discussions of these complex mixtures.

It is currently thought that the concentrations of oxygen and pollutants are nearly uniform throughout the ship due to the rapid recirculation of air between compartments [10,11]. An exception to this may be the differential distribution of aerosols between the engineer room and forward compartment. During the first decade of nuclear submarine operations, the total aerosol concentration (0.5 mg/m^3) was five times that of the aerosols measured in fresh country air and twice

that observed in large industrial cities. A "blue haze" of oil droplets (diameter ≥ 0.4 μm) was predominantly distributed in the engine room and occasionally irritated the machinists. Respirable droplets in the forward end of the ship (diameter ≤ 0.4 μm) were largely derived from cigarette smoke and predominantly distributed in the forward compartment. The deposition of these mists caused failures of electronic equipment and reduced the efficiency of cooling coils. Lower concentrations of aerosols were measured after the capacities of the shop's electrostatic precipitators were increased [10,12,13]. Recent measurements have shown a lower concentration of total aerosols in the forward compartment (0.1 mg/m^3) than in the engine room (0.2 mg/m^3) [14].

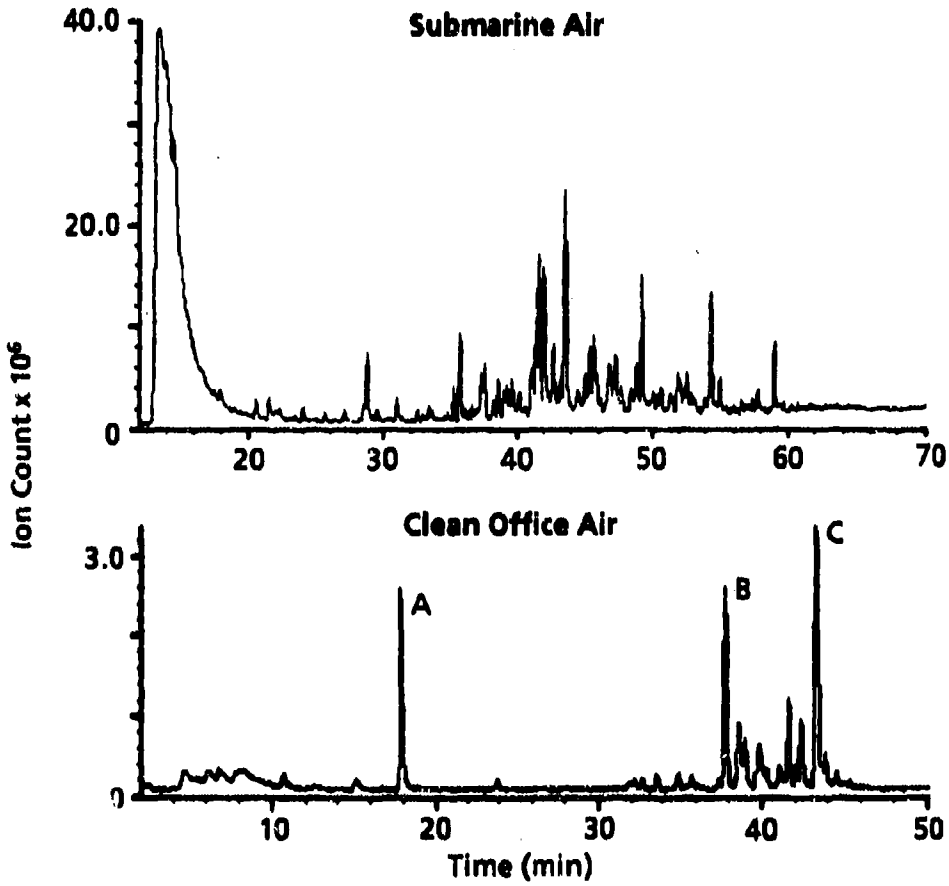

Figure 1. Total Ion Chromatograms of Submarine Air and Office Air. Both air samples were analyzed by chemists at the Naval Submarine Medical Research Laboratory. X axis: the time when different constituents of the air sample emerged from the gas chromatograph. Y axis: the count of ions generated by the mass spectrometer for each constituent emerging from the gas chromatograph. The concentrations of the contaminants are proportional to the areas of their characteristic peaks on the total ion chromatogram. Peaks A and B are markers for *n*-heptane (39 ppb) and indene (39 ppb), respectively. Peak C is a C11 hydrocarbon.

BIOMONITORING

Schaefer [15] postulated that the environmental stress and altered life styles that characterize occupational exposures to nuclear submarines cause a variety of subtle changes in organ function. Field studies implied that the 0.7 to 1.0% concentrations of CO_2 alter the permeability of red blood cells, increase the gastric acidity, raise the respiratory minute volume, produce cycles of metabolic and respiratory acidosis, and alter the calcium excretion in urine. Furthermore, the absence of sunlight may interact with CO_2 to reduce the urinary excretion of calcium. The resulting cycles of urinary calcium excretion were thought to indicate that bone serves to buffer the cycles of respiratory acidosis [15]. This prompted Messier et al. [16] to examine the hypothesis that submarine duty changes the bone mineral content as a result of exposure to the combined effects of atmospheric CO_2, demineralized water, lack of sunlight, and diminished physical activity. When submarine veterans (n = 10) were studied by total body neutron activation analysis, the total body mass of potassium and calcium were the same in the veterans as in a control population of civilians matched for age, weight, and height. In active duty submariners (n = 39), the bone mineral content of the left radius was measured by photon absorptiometry. The submariners had the same bone mineral content as a control population of civilians. The investigators computed the normalized bone mineral content of the active duty and retired submariners and found no substantial alteration as a function of submergence times between 6 and 52 months. The investigators concluded that repeated and prolonged exposures to the submarine environment did not produce cumulative changes in skeletal mass [16,17].

We recently studied the bio-uptake of VOCs by collecting samples of the expired breath from a submariner before and after an 82-day patrol. The subject, a nonsmoker, inhaled purified air through a Teflon® manifold and exhaled into a Teflon® bag. The expired VOCs were harvested for laboratory analysis by drawing the collected gas through Tenax® gas chromatograph-absorbent material. Total ion chromatograms of the submarine air and expired breath samples are shown in Figure 2. The major classes of VOCs in submarine air were the alkanes, aromatics, and O_2-containing organic compounds. The pre-patrol breath samples contained oxygenated-organic compounds and alkenes (principally isoprene) as the major classes of VOCs. Immediately after patrol, the predominant VOCs in expired air were the alkanes and halocarbons. The results of this trial study seem to suggest that occupational exposures to submarines alter the composition of VOCs in the expired breath.

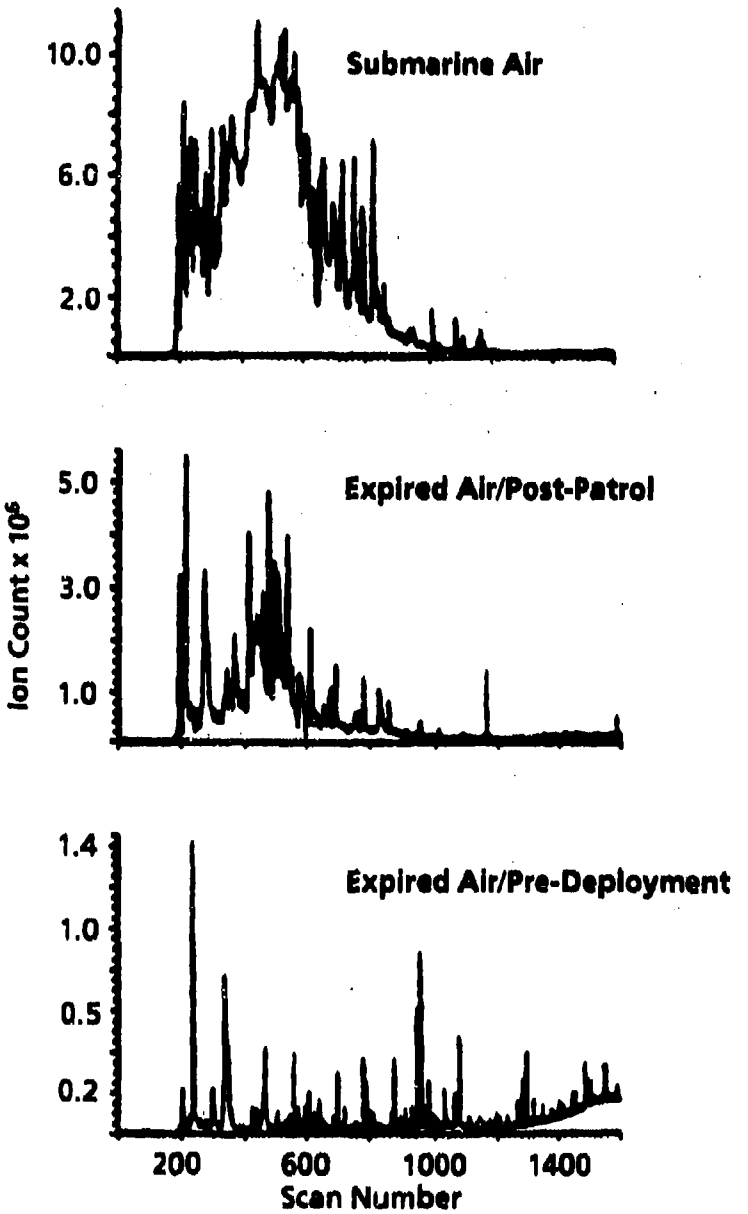

Figure 2. Composition of a Crewmember's Expired Breath. The axes have the same meaning as defined in the legend of Figure 1. The pre-deployment and post-patrol breath samples were collected outside of the submarine in comfortable rooms.

The bio-uptake of cadmium was studied [unpublished observation of Bowman and Bondi] by collecting samples of hair, blood, and urine from crewmembers during a two-month submarine patrol. Analyses of the air, blood, and urine yielded cadmium concentrations at or below the detection capability of the instruments. The hair samples of submariners had higher concentrations

of cadmium than observed in hair samples taken from nonsubmariners. Thirty days after patrol, the cadmium levels in hair were approximately back to those found before patrol. Of particular interest was the observation that cadmium contamination of the hair varied according to location of the submariner's watchstation aboard ship. The results indicated that (1) submariners may absorb cadmium as a function of the location of their watchstation and (2) the route of cadmium absorption may be through skin rather than the lung. More comprehensive studies are needed to define the uptake and metabolism of trace atmospheric contaminants by submarine crewmembers, particularly in relationship to the location of their watchstation aboard ship.

EPIDEMIOLOGY STUDIES

The morbidity and mortality of submariners have been defined by three epidemiological studies spanning the time period from 1963 to 1980. There was a decrease of the illness rates in submariners from the period 1963 to 1967 to the period 1968 to 1973. The reduced rates of illness occurred in respiratory, otolaryngologic, gastrointestinal, cardiovascular, urologic, and infectious diseases. These changes were attributed to improvements in the atmosphere control program resulting from an increased capacity of the CO_2 scrubbers, more frequent replacement of the carbon filters, and the use of more efficient catalytic burners [18].

Tansey et al. [18] reviewed the medical officers' reports from a decade (1963 to 1973) of 885 Polaris submarine patrols. The investigators tabulated illnesses resulting in absence from duty for one sick day or longer, and excluded deaths (n = 5) and medical evacuations (n = 37). Illness rates were defined as the number of new cases per 1000 man-days at sea. According to the *t*-test statistics, submariners had significantly lower rates of respiratory illness, gastrointestinal disease, and infections than did the control population of sea-going sailors. There were no significant differences in the rates of skin ailments and urinary tract diseases between submariners and sailors; however, submariners had significantly higher rates of cranial, systemic, and neuropsychiatric illnesses. Dental problems and headaches accounted for the higher rates of cranial illness in submariners. The systemic illnesses included cardiovascular problems, arthritis, and systemic viral infections. The rate of neuropsychiatric illness was higher after 1967, which was consistent with national trends in neuropsychiatric illness [18].

Hospitalization records were used by Burr and Palinkas [19] to evaluate the health risks associated with submarine duty during the time period of 1974 to 1979. The control group was a random sample of white enlisted men serving aboard frigates or destroyers. Age-adjusted hospitalization rates (i.e., the number of admissions per 100,000 man-years) were computed for major diagnostic categories and health problems considered relevant to the potential health effects of submarine duty. The relative risk of submarine duty was defined as the quotient of hospitalization rate for submariners divided by hospitalization rate for surface sailors. The health risks of submarine duty were lower for all major disease categories, including diseases of the lung, malignancies, and

infections. The submariners were at significantly lower risks for mental disorders, external injuries, genitourinary diseases, skin disorders, and musculoskeletal diseases. Submariners tended to be at lower risk for asthma and higher risk for diseases of the kidney, ureter, and heart, but the relative risks were not significantly higher as determined by the use of 95% confidence intervals. Possible reasons for lower hospitalization rates of submariners were stringent medical screenings of candidates, their higher level of education, difficulty with conducting medical evacuations from deployed submarines, and the transfer of medically disqualified men from submarine duty. In the investigators' opinion, the health status of enlisted men was not adversely affected by submarine duty [19].

Ostfeld et al. [20] reviewed the mortality records of deceased crewmen to determine the risks associated with occupational exposure to trace contaminants of submarine atmospheres. The cohort consisted of 77,123 enlisted men and 8,628 officers who were on submarine duty between 1969 and 1981. Less than 2% of the veterans were unavailable for follow up after discharge from the Navy. Submariner mortality was evaluated by use of the standardized mortality ratio (SMR), which is the ratio of deaths observed in submariners to deaths expected from the mortality rates of the U.S. male population. Confidence intervals of 95% were calculated for the SMRs and inferences of significance were made when the confidence intervals excluded the SMR-value of 1.0 [20].

By the end of 1982, there were 351 in-service deaths and 527 out-of-service deaths. The SMRs for in-service deaths from all causes were considerably less than 1.0. These low mortality rates probably resulted from careful health screening of new recruits and the prompt discharge of medical disability cases from the Navy. The out-of-service death rates were lower than expected for diseases of the heart, lung, and liver. The SMRs in Figure 3 show that the veterans had the same number of deaths as expected for U.S. males from external causes (i.e., accidents, suicides, and homocides) and cancer. The accidental deaths of enlisted veterans were higher among those with history of demotions or duty aboard fast-attack submarines. There was clustering of cancer deaths in the year immediately following discharge from the Navy for medical disability. The rates of mortality from neoplasms of the bone, connective tissue, brain, and central nervous system were collectively 1.34 times higher than the death rates of the general U.S. male population (Figure 3). A log-linear model of death rates showed that length of service was a significant predictor of the cancer death rate, particularly death due to lung cancer. Unfortunately, data were not available on the risk factor of smoking. Specific occupation aboard the submarines was only marginally predictive of the cancer death rate, being relatively higher among technicians (85% of the enlisted cohort) than among administrative personnel. As a matter of speculation, Ostfeld et al. suggested that the contamination of submarine atmospheres has decreased over the years due to improvements in the air filtration system. Therefore, the probability of cancer induction from submarine duty-related exposures by this speculation would be much less now than in the past [20].

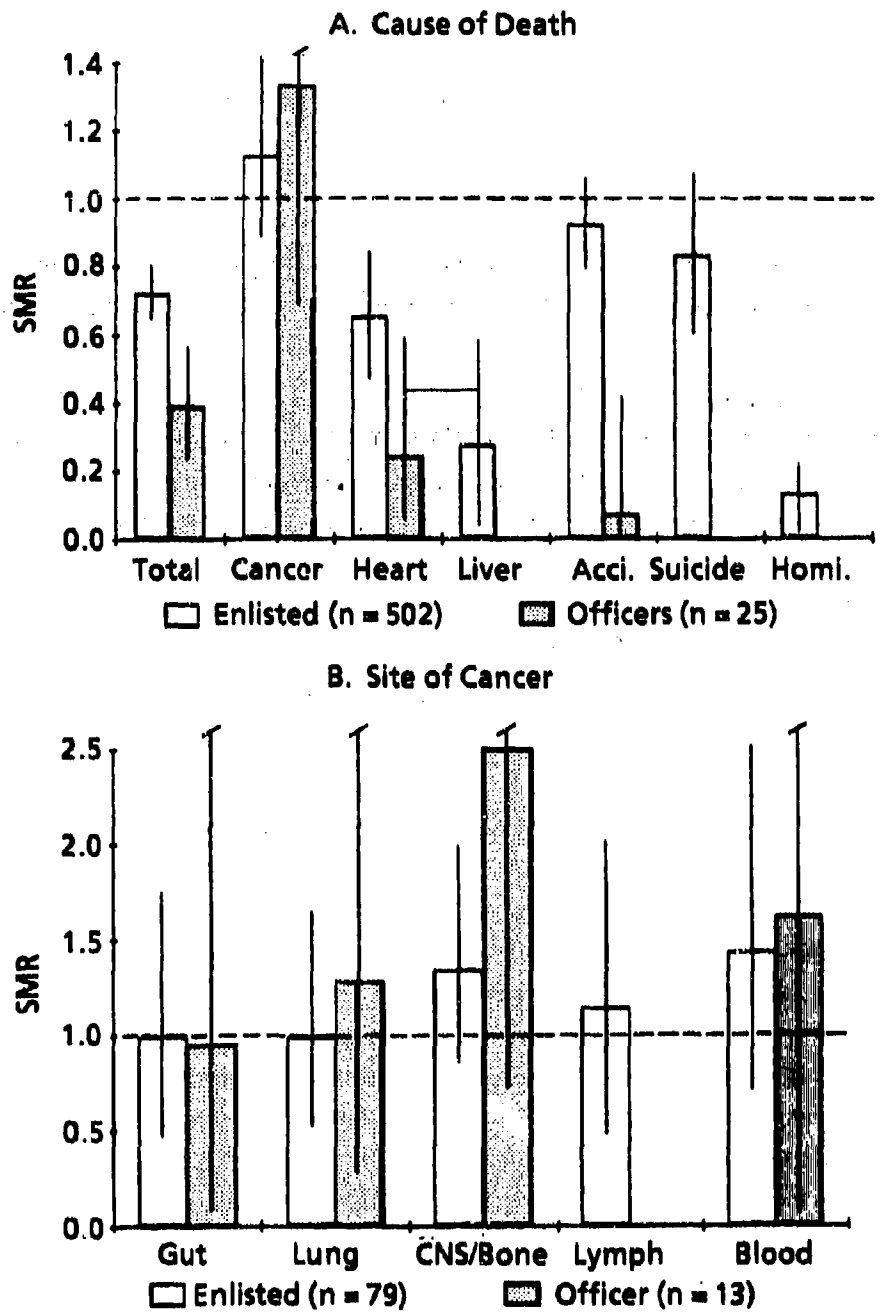

Figure 3. Mortality Data of Submarine Veterans. The data are taken from Ostfeld et al. [20]. The SMR is the ratio of observed to expected mortality rates for submariners. Each bar is intersected by a vertical line, which denotes the 95% confidence interval for that particular SMR. ABBREVIATIONS: Acci. is accidents; Homi. is homicide; and CNS/Bone refers to tumors of the central nervous system, bone, and connective tissues.

CURRENT ISSUES

Eight decades of improvements in submarine atmospheres have diminished the concerns of environmental engineers for acute toxicity, explosive gases, corrosion of equipment, and unsuitability for habitation. However, atmospheric monitoring must continue to ensure that these problems do not recur with the introduction of new materials aboard ship. There is still the problem of selecting the most effective methods for monitoring submarine atmospheres with respect to the health of the crews [10].

The results of medical studies indicate that the atmospheres aboard today's submarines are not chronically toxic to the crews. In view of current developments in understanding the toxicity of complex mixtures [9], more work is needed to ascertain the biological activity of submarine atmospheres. A longer follow up of submariner mortality studies would be advantageous in assessing the risk for diseases of long latent periods.

It is doubtful that atmospheric contaminants are uniformly distributed throughout the submarine in view of the different densities of aerosols between the forward and the engineering spaces of the ship. This raises the possibility that crewmembers differentially absorb contaminants as a function of the location of their watchstation.

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STUDIES IN THE SYNERGISTIC HEPATOTOXICITY OF CARBON TETRACHLORIDE AND TRICHLOROETHYLENE OR CHLOROFORM IN MALE F-344 RATS

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(Manuscript Not Submitted)

SESSION II

PANEL DISCUSSION

Dr Paul Feder (Battelle Columbus Labs): For Dr DeMarini. In your diagram you showed fractionation going from the top down. That would be appropriate if you thought that there might be several substances interacting together that gave you a toxic effect that you may not be able to see separately. If there were one or two major opponents, wouldn't there be the possibility of "diluting," so to speak, the effect of the bad guys with the relatively innocuous parts, or would you also want to start from the bottom up and test relatively pure fractions and then combine things where you found toxicity?

DeMarini: That is how you have to do the process. To get those relatively pure fractions, you have to start producing relatively crude fractions and then successively purify those. So it makes good sense that you are going through that process to do bioassays on those fractions, as you go along. The real meat of the data certainly comes at the end with the more purified fractions. Depending on how good your fractionation methods are, you begin to generate fractions that are more chemically homogeneous, such as aromatic amines, nitroaromatics, aliphatics, and then when one is identifying interesting biological activity in such a fraction it becomes much more amenable to analytical chemical analysis than fractions that are far more chemically complex and contain a large variety of different classes of chemicals. What you say makes sense, but in practice one has to work down to a more pure set of fractions.

Dr George Anstadt (Miami Valley Hospital): I have a question for Dr Sipes that I have often wondered about and never had the right person to ask. Of the other shorter-acting barbiturates than phenobarbital for instance, particularly pentabarbital, does it have the same potentiating effects or any degree of potentiating effects on carbon tetrachloride or things like that?

Sipes: Usually the shorter the acting duration of a barbiturate, the less inducing activity it has, but I think with pentabarbital, given in repeated doses, you could probably show some potentiated response because I think it can induce; it is just not as potent as phenobarbital.

Anstadt: Is there any documentation of the inducing capability of pentabarbital?

Sipes: I think there is evidence of its inducing capability, yes. But again, phenobarbital is a classic and it is more potent. Some of the other barbiturates, I think secobarbital, can actually destroy P-450 so it actually can have an inhibitory or antagonistic effect. It has an allyl group on it that destroys certain hemes, so, with some of these agents, you can get mixed effects. But I think the important thing to remember is the longer the duration of action, the greater inducing capability they have. The one

thing that I didn't point out is that there are many, many examples of environmental compounds that patients may be exposed to that have inducing capabilities.

Anstadt: I have one other question, if I dare ask it. It is a little bit irrelevant, but I have heard the comment that if the vaporizers were as good years ago as they were in the advent of halothane that chloroform would have been just as good of an anesthetic, if the vaporizers would be available for it, in terms of the hepatotoxicity of halothane versus chloroform. Do you have an opinion about the relative hepatotoxicity of chloroform versus halothane?

Sipes: Yes, I sure do. I certainly would not choose to be anesthetized with chloroform; I would much prefer halothane. We have worked on that compound for 15 years and it is tough to make it hepatotoxic. I will point out that chloroform, at least in my estimation, is not that hepatotoxic until you start doing a couple of things, including enzyme induction or glutathione depletion, and that is very easy to do in animals and it is very easy to do in humans. With halothane you have to work at it, and I will be happy to tell you later what our current feelings are in the mechanisms of halothane-induced injury, but with chloroform, you can reproducibly show on a variety of different animal models that it can be hepatotoxic.

Dr Rory Conolly (NSI): I would like to ask a question that really is addressed, I guess, to the whole panel. It is kind of a philosophical question about the studies of mixture toxicity. It is not meant to criticize any of the presentations today because they were really quite excellent with respect to the work that is being done on mixtures. But it seems to me that if mixture toxicity studies are going to be anything more than purely descriptive, that is, the sort of modern-day analog of the 1960s studies where we counted the number of legs and divided by four, that inevitably it leads you down to the question of what are the mechanisms of the components of the mixture, and understanding those mechanisms gives you some basis for understanding the kinds of interactions you are seeing and maybe making predictions about what you might expect. But given that it is true, you are led back to where we are with single compounds today, which is that we are all struggling to try and understand single compounds and in one of two cases doing fairly good jobs of it, but in most cases really having very little idea at all of what the mechanism of a toxic chemical is. So I guess my question is, where can we seriously expect mixture toxicity studies to go, given the limitations of our techniques and our understanding of even single compounds?

Mehendale: I believe the points you have made are well taken and I think we ought to pay greater attention to whether or not we are unraveling additional mechanisms from this combination of toxicology. Our own study with chlordecone, carbon tetrachloride and chlordecone, chloroform and chlordecone, and a few other compounds has suddenly led to a new approach to mechanisms. We would like to think that it is a new mechanism. For example, if chlordecone is a neurotoxic compound

when given in sufficient quantities, and when it is given at low levels, there is no indication of any kind of toxicity anywhere. We know how to measure it in that animal. And now, on top of that, the animal is exposed to a tiny bit of carbon tetrachloride, and enormous amplification of toxicity occurs, and it does not occur by increased metabolism or increased lipid peroxidation, increased covalent bonding, or any number of these putative mechanisms. Then we have to look for a new mechanism and that is why we look for it, and we believe the mechanism is one of interfering biology of the cell. Clearly, having the animal eating food containing chlordecone interferes with that biological process, which is essentially protective mechanism. That, I think, is a completely normal mechanism, whether it stands up to the test that we will pursue in the future or not, certainly it has led us to a new mechanism. I think this is a direct result of the combination toxicology or toxicology of mixtures. Basically, what that concept says then if you want to go a step further, is that you can simply interfere with repair and not have to have additional or greater putative mechanisms to explain toxicity. What I am saying is, it is a new mechanism, a new way of thinking, and I believe that this approach has led to that kind of thinking.

Conolly: I take your point and I think it was well made, but I am not sure that you really answered the question that I was asking. The point you made is that by studying extras we can learn about mechanisms, perhaps from a new approach that we have not taken before, and that is great, because I personally think that is what we need to know about, are mechanisms, but it seems to me that the whole question of mixtures is really out there somewhere in the future until we really know enough about mechanisms that we can start putting them together in complex ways and making predictions about mixture toxicity.

Yang: Rory, I think that is very true, I totally agree with you, from the point of view of our 25-chemical mixtures. Two years ago, I showed an equation on a board – at 33,541,000 or so combinations – so it is impossible you could add everything up, but on the other hand, I am looking from the point of view that we do not know anything about health effects of these things. The first step is that if we show some effect, which we are very surprised to see really, and then maybe the mechanism of such effects may not be just of one chemical, it may be collectively because of physicochemical reasons, that all of those have got to be there. So you can look at it from one point of view that this mixture is actually one compound. Then you can do the mechanistic studies we are interested in doing. Right now, we are doing a study on what is the pretreatment of this chemical mixture having to do with, for example, the pharmacokinetics of carbon tetrachloride, pharmacokinetics of methylene chloride, and you can expand that further to DNA bindings, and that sort of thing. Consider that as a "phenobarbital." Now I am not as pessimistic as you are; in fact, I think if I, in my lifetime, cannot answer this question, somebody later on will. You know, we are just providing a piece of the mosaic. In fact, I am so optimistic that I have talked to Mel Andersen, and I

said "what is the possibility of doing modeling on 25 chemicals with mass balance equations interconnecting all those chemicals together?" and he said "Theoretically, it is do-able," so I am thinking that even down the road maybe we can do some predictions, if we have enough material, knowledge, and so on.

Anstadt: May I make one comment? I want to boohoo everything you have just said because I want to talk about something other than binary mixtures, even 25 mixtures of chemicals mixed together, and that is complex mixtures. I think that it is rather hopeless at this point to realistically talk about discovering mechanisms with such complicated mixtures when we cannot understand the mechanisms of just two compounds mixed together that have been studied for 30 years, and I think that it is important to not focus on that question when it comes to complex mixtures, but to get over it, to get on with the business of studying complex mixtures, fractionating them. There have been studies where the fractions have been mixed together in various ways. Reconstituted mixtures have been studied. There has been interesting kinds of synergisms and antagonisms observed from those kind of studies and I must tell you that of all the studies done on interactions in the field of genetic toxicology, after close to 15 or 20 years of work, no first principles yet have been identified and I don't think any are likely to be identified in the near future because of the complexities of the systems, the complexity of the endpoint, and the complexity of the chemical world. I don't think it means we should not keep looking; I think it is very important. But when it comes to real-world complex mixtures, I think that is not exactly a question whose time has come.

Dr Clay Frederick (Rohm and Haas Company): What I found particularly pleasing was the discussion of the effects of Vitamin A and also the effects of basically brain-hormone type effects and the relationship to xenobiotic metabolism. The reason is because I think much of the work I have seen on mixtures seems to have been driven by the EPA priority chemicals and the interactions of the halogenated hydrocarbons and all, and I feel like we are running down a narrow road in the study of mixtures and interactions of things. We are basically talking about differences in the interaction of metabolism, either inhibitors of P-450 or enhancement, induction of this activity, but when we start talking about different physiological mechanisms drawn from very different compounds, very different roots, and how they may affect things, I think we may be doing more to reflect the real world. I think if there is one hope, if you will, to the study of the interaction of mixtures, it is to talk about class physiological effects. In other words, materials that may, in fact, induce Kupffer cell activity may be a general effect on other compounds, that may be a general mixture effect. That is the only real hope that I see in understanding this mixture game; otherwise, you are going to have an infinite number of possibilities if we do just mix and match in twos, and threes, and fours, and thousands.

Anstadt: If it is any consolation, there are now studies going on in mutagenesis that are mixtures of mixtures and this is the field of antimutagenesis. Obviously, as a lot of you know, extracts of many, many vegetables, especially cruciferous vegetables, contain a lot of antimutagenic activity and so there is now a whole series of studies, mixture studies, combining those extracts with mutagenic complex mixtures, in looking for inhibitions, and they get very complicated.

Dr James Trosko (Michigan State): I was recently at an international conference and I would like to share with you something that I think has bearing on Dr Sipes's and Dr Mehendale's and the whole panel's discussion of mixtures and toxic effects. The first thing is we have to remember when we study single chemicals either *in vitro* or in animals, we are really not studying single chemicals, because a single chemical in a tissue culture is interacting with growth factors, which tend to give spurious results from one laboratory to another if they don't use the same lot. And in the animal this may explain why a single chemical, either at a different developmental stage, or different species, or different sex gives different results, because it is interacting with endogenous chemicals that are different at these different stages, tissues, and sexes. But more importantly, I think it gets down to understanding mechanisms. The problem is, if we do not understand mechanisms responsible for single chemicals, how can we understand it in complex mixtures? What are we talking about when we are talking about mechanisms? One speaker talks about mechanisms at the molecular level, DNA repair for example; another is talking about biochemical mechanisms, glutathione scavenging or enzyme induction; another person is talking about cellular mechanisms, or cell-to-cell communication; another is talking about physiological mechanisms like neuroendocrine effects. So we have to be careful of which level of biology we are talking about, not that they are independent of each other, because they are, in a hierarchy, all interrelated, but finally, one mechanism involved in many chemical toxicities is, in fact, disrupting the basis for cellular homeostasis that is necessary in a multicellular organism, namely gap junctional communication. Within that level we now know there are five different mechanisms controlling how a chemical blocks cell-to-cell communication. Having identified those five, it is interesting now that we can determine that a chemical such as phorbol ester blocks gap junctions by activating protein kinases, we know the mechanism. We can then take DDT, which we know blocks gap junction of communication, and we now think we know the mechanism there – it enhances intercellular calcium. Both of these mechanisms block gap junctions; that is, what would be the prediction if we added the two together? Would they be additive, would they be synergistic? We know something about PKC – it is a calcium phospholipid-sensitive enzyme. Therefore, if we add these two chemicals together, they may be synergistic. On the other hand, if you know the mechanism by which this particular phenomenon, gap junction of communication, is effected, you can now do additive studies. Add two chemicals that modulate gap junctions by raising calcium, like DDT and dieldrin, or ketone by the way, and, in fact, you get additivity rather than

synergism. We also now know that there are some chemicals increasing cell-to-cell communication as well as others that block it. A Swedish group at Karolinska added those kinds of chemicals, knowing how each individual chemical modulated gap junction, and they were able to antagonize it.

So what I am saying is that if you identify the level by which the chemical presumably is affecting the toxicology, the mechanism at that level, the cellular and the biochemical, you might now have a chance to get at predictability of these complex mixtures. Finally, as a point of information and a fact that you might be able to run with, Dr Mehendale, it might be interesting to know that when you partially hepatectomize those livers, you are down-regulating gap junctions, and, through the gap junctions, by the way, glutathione can flow back and forth. So by isolating the cells with the partial hepatectomy you down-regulate the gap junctions, and the cells cannot talk to each other; they can neither protect each other nor can they share the damage. My suggestion is, under those studies in which you have observed these kinds of synergisms and protective agents, you look and see what happens to the gap junction. All I am saying is these are different levels by which mechanisms can be studied and I think that you and others up there have studied at different levels but you have not looked at the one that ties the whole organism together, and it is this interaction, which Dr Sipes's work is now clearly pointing to.

Sipes: I think that what you have to keep in mind again is the question you are asking: "Is everybody looking for a basic fundamental mechanism?" If everything gets down to that level, fine, but you also want to know what is the triggering event and is the triggering event in the CNS or is it in the Kupffer cell, or is it in the big toe, and just because everything comes down and it effects one of the particular gap junction proteins, in the long run, that is fine, if you know it is a common mechanism. I think you want to know where the initiating event is that results in the interaction; and I think that is the way some of the talks were focused today. And the other thing, talking about complex mixtures, I hope that everyone realizes that you can have things that up-regulate, down-regulate, inhibit, enhance, and that is going to be very complex. In approaching the question head on, for specific hazardous waste sites or whatever, that may be one way to ask the question. I think that I want to underscore what was said. I was involved in some of the Love Canal studies and when they faced it head on and said we are going to expose the animals to this mixture and see what happens, I thought that was a novel way of doing it. Many years ago, people would not have wanted to fund that because we did not know how one chemical worked. These mixtures are going to change, too, and the components in them can change, so it is a tough issue.

Mehendale: Thank you, Dr Trosko, for your comments. One, I completely agree with you that you have to go level-by-level to look at the mechanism. In our own case, identifying that there is something wrong with the biology of cell division allows us to reach that one level and therefore open up that cell and look at the molecular biology of why one cell divides and one cell doesn't.

Concerning the gap junction issue, we have not really looked at that; we were aware that the gap junction will be down-regulated or less by partially hepatectomized liver cells. Many of these cells are newly divided and perhaps would not have had the time to double up gap junctions. Glutathione levels in those cells are at or slightly above normal levels – it is only that biochemical reason to be involved – and second, carbon tetrachloroide toxicology does not involve decreases of glutathione as a primary event. Later on, as the toxicity progresses, it does. Therefore, that particular biochemical would have something to do with it. Perhaps that is an issue we need to look at in the future. However, what we have done so far allows us now to go into the cell and ask some specific questions and therefore go at another level of mechanisms.

Dr Bernard Schwetz (NIEHS): I think there are two important approaches that we as scientists are using to look at this problem of mixtures. One is to look at the toxicity in a descriptive sense of what these mixtures do to animals, but that is an endless process because there is an infinite number of mixtures and it is going to be a real slow process to make much of a dent in that, unless we are really surprised by either the lack of toxicity or a significant toxicity from these mixtures at environmental levels. The other one is what has been well-described today with some very good examples of looking for mechanisms for which these interactions occur. But understanding mechanisms has not been an easy and rapid process. It may take a long time for us to understand the mechanisms of some of these simple mixtures and then begin to understand how they operate at an environmental level, or with other components of mixtures. Because in the meantime we are assuming an additivity model for predicting the toxicity of these mixtures that are out there in the environment, are there some studies that you would conceive being useful that could be tested now to challenge the additive model with mixtures without knowing the mechanism of action or interactions?

Mr Kurt Enslein (Health Designs Inc.): One should be daunted by this wide array of varieties of complex mixtures in the environment. When you at least look at the genetic toxicology of the wide array of complex mixtures that have been studied, it turns out that they actually fall into rather interesting, somewhat discrete classes. They are not as diffuse and wide as one would imagine. Combustion emissions tend to fall in a particular potency range in terms of their mutagenic potency and their carcinogenic potency based on mouse skin tumor studies. Certain other kinds of mixtures, such as surface water and drinking water concentrates, tend to also fall within a certain narrow range, usually just two orders of magnitude of mutagenic potency; they have not really been studied for carcinogenic potency yet. And so, although there is a wider range of mixtures out there, they may not be as diverse as one might think. It sheds some ray of hope, I think, in beginning to get a handle on what are the kinds of interactions going on and what are the kinds of differences that we might expect to find between certain types of mixtures. They are not all across the board. They do seem to fall in certain categories.

Yang: As one of the most important aspects in terms of involvement of pollution, mixture exposure to people is at low level and long-term. For that, I would like to launch the concept of "promoter" or "enhancer," this is, in a generic sense. Promoter is not limited to carcinogenesis. It could be something that would promote any toxicity. In other words, as I presented this afternoon, you may have a situation where you are exposed to a chemical mixture of some sort. Clinically and by conventional toxicity indices, the animal will be normal, and yet, upon a certain challenge, be it drug intake or accidential exposure, joint toxicity might ensue. That is the kind of study, which, in my view, is very important, and in Dr. Sipes's lecture, he was talking about a combination of events leading toward joint toxicity, and I really like that idea very much. I think it is not just a single mechanism, but it would be a combination of mechanisms toward one common endpoint.

Mehendale: Just a brief comment. Suddenly, we already have been doing complex mixtures studies whether we want or not. Diesel exhaust and cigarette smoking are perhaps the two most illustrious examples of mixture toxicology and suddenly those are very important, relevant studies. Some compounds are going to antagonize, some compounds are going to increase, but to some extent it is still an increase, and in this case, increases chances of cancer. Therefore, I think even if we don't get mechanisms – getting at mechanisms is going to be painfully slow – I think we still benefit from doing these studies and the studies will obviously improve as we learn the not-so-optimistic studies we have just completed.

Dr Ron Wolff (Lilly Laboratories): I just want to return to what you said in the beginning about keeping an open mind on the subject. I think that is particularly important with complex mixtures and I think I want to share what I think are a couple of insights I gained in some work with James Bond and Joe Moderly at the Lovelace Laboratories before I left, related to diesel exhaust toxicology. We had done work for a number of years using a model system of benzo[*a*]pyrene inhaled with associated particles and we, along with previously Saffiotti and Nettesheim and others have shown that when these materials were administered to the respiratory tract together, benzo[*a*]pyrene was retained in the lung for much longer time periods than if it was on carbon particles or other particles, the inference being that longer retention of the material in some way led to greater toxicity. We did all those experiments and then in the last year we looked at DNA adducts after exposure to pure benzo[*a*]pyrene and benzo[*a*]pyrene associated with carbon particles to use that as an index of genotoxic damage, and found, as we had before, that the total ^{14}C label over the 12-week exposure was at least a hundredfold higher when material was associated with particles, but the DNA adduct levels were not different between a pure benzo[*a*]pyrene exposure and a combined particle exposure. This led us to re-think things a little bit in terms of the realm of carcinogenicity. I think we do need to keep open minds on these complex problems and keep digging at things from the available facts.

Major John Latendresse (Navy Toxicology Detachment, Wright-Patterson Air Force Base): I wanted to ask Dr Sipes if there was any particular morphological pattern related to the hepatocellular necrosis in the systems that you studied showing Kupffer cell activation?

Sipes: You mean relative to the response to carbon tet itself or just to the Vitamin A?

Latendresse: I was just wondering if, with the Kupffer cells actively involved in the damage, does that provide a particular morphologic pattern of degeneration or necrosis in the liver?

Sipes: Not that I can tell you. I am certainly not an expert in the area. You are going to hear much more about the role of Kupffer cells in injury. I know there are several people working on this particular area now. In some cases, Kupffer cells become involved because they are recruited into the liver from plasma and they become a resident macrophage and they may be recruited in. It reminds me of some of the work from Rutgers with Debbie Laskin. She is looking at a model where with the acetaminophen she gets no necrosis at 24 h but between 18 to 24 or 36 h there is recruitment of Kupffer cells and then there's probably this oxidative burst that displays the greater hepatotoxicity she sees at 48 h. In our system we are not recruiting, we are activating Kupffer cells. We cannot see any increased number; we do not see an infiltration. So essentially what we see is basically an augmentation of the carbon tetrachloride-induced injury. There may be an underwriting role for the Kupffer cells there that we are just going to start appreciating. I actually had a question for Hari who was talking about the fact that these cells have to divide in his ketone model, that he needs mitosis, and do you have to have clearance of dead cells in order to trigger cell division, do you know? I do not know if I answered your question, but we can see no difference histologically. If you gave a small dose of carbon tet-treated Vitamin A to an animal, and you gave an equivalent dose that would produce the same degree of toxicity of carbon tet, you know a very large dose, with those equivalent degrees, I cannot tell any difference. The only change at the light level that we can see, now that we know that Kupffer cells are activated, is they are more prominent in the sinusoids of even H&E-stained cells, and if you stain specifically they just light up. Or, if you give carbon products or other agents that they will take up, you can see them more readily, but there does not seem to be a large increase. I do not see a marked morphological difference.

Dr Ugis Bickis (Queens University): Of course the classic example of promotion of carbon tet hepatotoxicity relates to ethanol, and Dr. Sipes mentioned that this could be due either to the stimulation of Kupffer cells and both the inhibition of Kupffer cells, if I understood correctly, depending upon whether it was an acute dose or a chronic dose. I was wondering if he could reconcile for my benefit how those two factors could both lead to the promotion of the effect, or secondly, how Dr Mehendale's model might also account for the interaction between alcohol and carbon tet?

Sipes: I guess what I was trying to say was that what we have been able to find is that if you treat Kupffer cells with ethanol and measure portal blood levels, you have slight activation but as you increase the dose of alcohol to say three or four doses over a two-day period, 4g/kg or something of a pretty large dose, Kupffer cell activity can be decreased. We also are able to show that acetaldehyde severely depresses Kupffer cell function, so I guess what I was trying to say is that if you have a role where you have an activated Kupffer cell that could participate an injury, you could possibly potentiate injury at that stage. If you needed the Kupffer cell there for its normal phagocytic function and it was not functioning and you had endotoxins or other agents that would be coming up from the intestinal tract where you are depending on Kupffer cell function, and they may then reach the liver in much higher concentrations than they would if you had active Kupffer cells and how that would impinge on the liver's function, I don't know. I was just trying to make the issue that it comes back to the same thing with metabolism. In some cases you can protect the liver by enzyme induction and in other cases you can't, if it is an activated species, make the liver more susceptible to injury. The one thing about alcohol is that it has been very clearly shown that it induces a particular P-450 that activates a large number of compounds. We actually tried with our Kupffer cell model with acetone, which we showed a long time ago, that acetone is a potent potentiator of carbon tet injury, we thought that maybe we were getting Kupffer cell effect there, and we tried to block that and it had no effect. We think that the acetone effect is probably coming back to that it is inducing a particular P-450. I think the whole thing is really complex, and I think people working in the alcohol field would probably relate or understand it as a complex issue, and I don't know if people were talking about repair processes and how alcohol could influence that. I should say, I was just talking about acute hepatocellular injury and not even thinking about the long-term consequences of chronic exposure to Vitamin A, for example, which may lead to cirrhosis in its own right or fibrosis, so it was looking very acutely at a response.

Mehendale: If I may just briefly respond to that question as it related to some of our work. When we learned about the Kupffer cell activation with acetaminophen, we looked for some indication that perhaps that might be happening with our chlordecone-carbon tet interaction, and we couldn't find any indicators in support of that with that interaction. However, recently we have done some studies where we have taken a homologous series of alcohols and looked at potentiation of carbon tet toxicity. We have not published that work; it will be presented at SOT. The rationale there was when we looked at the literature for a lot of these kinds of studies, save for a few exceptions, people had used rather large doses of alcohol in those interaction studies, where those alcohols themselves were somewhat toxic, either after repeated administration or single administration. We wanted to look at combinations where the alcohol by itself would not be toxic by measures of classic liver injury, and then look at whether carbon tetrachloride toxicity will be enhanced. And the findings are that the

alcohols are falling almost distinctly to two groups. We have not covered all of the structures; we were looking at about 10 different alcohols, starting from one carbon methanol to 20 carbon icosynol. We find that some alcohols increased liver injury under those circumstances, relatively low doses of carbon tet also, and also increased fatalities from it, so if you leave those animals alone, in fact, more numbers go on to die. The other alcohols in the other group increased liver injury but, if you leave them alone, nothing ever happens to those animals. That is intuitive to me that there is something interesting there. Why in both cases is there increased liver injury, but only in some cases the animal will actually go on to die from it, so that is not a reversible process? And in some cases, it is reversible. The reason I pointed these studies out is we have looked at the liver sections and we see a lot of lymphocyte infiltration, much more than we have seen in our carbon tet interaction. We don't know what that means but are interested in those things and what those cells are doing. We are looking at part of the answer to your question.

Dr Bruce Stuart (A.D. Little): The question of complex mixtures has been revolving around one of great interest to me and that is diesel engine exhaust. It started with uranium miners back in the mid-60s. Let me just offer the fact that although it is a very complex mixture, if you examine the source term you obtain a mixture that is representative of the real world. This was found in studies that we promoted from the uranium mines analyzing what was actually happening in terms of the ratio of carbon monoxide, particulates, NO_X, the aldehydes, and aliphatics, and then reforming this by a modified diesel engine in the laboratory, were able to produce lesions within the lung that progressed into vesicular emphysema, sequestration of pulmonary alveolar macrophages in interstitial regions, and set the stage for the later development of pulmonary carcinoma. I mentioned this because other studies done with perfectly operating engines that were not under cycles of load and RPM did not produce the quantities of particulate with associated PAH that are found in the real world.

Enslein: There are now five ventilation studies with diesel exhaust and they are very compatible with the short-term bioassay results in terms of the relative carcinogenic potency calculated from them.